Experimental Gerontology 52 (2014) 55–69

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Joint inhibition of TOR and JNK pathways interacts to extend the lifespan of *Brachionus manjavacas* (Rotifera)

Terry W. Snell *, Rachel K. Johnston, Brett Rabeneck, Cody Zipperer, Stephanie Teat

School of Biology, Georgia Institute of Technology, Atlanta, GA 30332-0230, USA

ARTICLE INFO

Article history:
Received 19 October 2013
Received in revised form 17 January 2014
Accepted 20 January 2014
Available online 29 January 2014

Section Editor: T.E. Johnson

Keywords:
Lifespan
TOR
JNK
Mitochondria
Lysosomes
Swimming

ABSTRACT

The TOR kinase pathway is central in modulating aging in a variety of animal models. The target of rapamycin (TOR) integrates a complex network of signals from growth conditions, nutrient availability, energy status, and physiological stresses and matches an organism's growth rate to the resource environment. Important remaining problems are the identification of the pathways that interact with TOR and their characterization as additive or synergistic. One of the most versatile stress sensors in metazoans is the Jun-N-terminal kinase (JNK) signaling pathway. JNK is an evolutionarily conserved stress-activated protein kinase that is induced by a range of stressors, including UV irradiation, reactive oxygen species, DNA damage, heat, and bacterial antigens. JNK is thought to interact with the TOR pathway, but its effects on TOR are poorly understood. We used the rotifer *Brachionus manjavacas* as a model animal to probe the regulation of TOR and JNK pathways and explore their interaction. The effect of various chemical inhibitors was examined in life table and stressor challenge experiments. A survey of 12 inhibitors revealed two, rapamycin and JNK inhibitor, that significantly extended lifespan of *B. manjavacas*. At 1 μM concentration, exposure to rapamycin or JNK inhibitor extended mean rotifer lifespan by 35% and maximum lifespan by 37%. Exposure to both rapamycin and JNK inhibitor simultaneously extended mean rotifer lifespan by 65% more than either alone. Exposure to a combination of rapamycin and JNK inhibitors conveyed greater protection to starvation, UV and osmotic stress than either inhibitor alone. RNAi knockdown of TOR and JNK gene expression was investigated for its ability to extend rotifer lifespan. RNAi knockdown of the TOR gene resulted in 29% extension of the mean lifespan compared to control and knockdown of the JNK gene resulted in 51% mean lifespan extension. In addition to the lifespan, we quantified mitochondria activity using the fluorescent marker MitoTracker and lysosome activity using LysoTracker. Treatment of rotifers with JNK inhibitor enhanced mitochondria activity nearly 3-fold, whereas rapamycin treatment had no significant effect. Treatment of rotifers with rapamycin or JNK inhibitor reduced lysosome activity in 1, 3 and 8 day old animals, but treatment with both inhibitors did not produce any additive effect. We conclude that inhibition of TOR and JNK pathways significantly extends the lifespan of *B. manjavacas*. These pathways interact so that inhibition of both simultaneously acts additively to extend rotifer lifespan more than the inhibition of either alone.

1. Introduction

Aging is influenced by the expression of hundreds of genes and their interactions with the environment (Magalhaes et al., 2012). A major challenge in aging research is to understand how the environment modulates aging gene expression, specifically what are the effects of interventions like diet, lifestyle, supplements, and drugs on aging (Lopez-Otın et al., 2013). The intention is to selectively manipulate signaling pathways to slow aging and produce healthy life extension.

Certain environmental manipulations like diet affect the rate of aging in most animals investigated. All organisms have evolved genetic programs to deal with stress like starvation, and modification of some of these can postpone aging. Nutrient sensing pathways, like those involving IGF, TOR, SIRT, and FOXO genes, often play key regulatory roles in aging (Haigis and Sinclair, 2010; Katewa and Kapahi, 2011). Aging researchers seek to understand how the expression of these key genes can be manipulated by diet and drugs to produce life extension and which genotypes are most likely to benefit from such interventions.

In a variety of model animals, the target of rapamycin (TOR) pathway modulates lifespan (Sharp, 2011; Stanfel et al., 2009). TOR is a nutrient responsive kinase in the glucose sensing pathway, conserved in yeast, invertebrates and mammals (McCormick et al., 2011). A number of environmental conditions reduce TOR signaling, including dietary restriction. Reduced TOR signaling in yeast mimics dietary restriction by shifting metabolism from fermentation to respiration (Kaeberlein, 2010). In eukaryotes, mTORC1 is a central modulator of cell growth whose expression is regulated by amino acid availability (Jewell and Guan, 2013; Yuan et al., 2013).

* Corresponding author at: School of Biology, 311 Ferst Drive, Georgia Institute of Technology, Atlanta, GA 30332-0230, USA. Tel.: +1 404 894 8906; fax: +1 404 385 4440.
E-mail address: terry.snell@biology.gatech.edu (T.W. Snell).

http://dx.doi.org/10.1016/j.exger.2014.01.022
0531-5565

TOR is linked to other invertebrate aging and nutrient-responsive signal transduction pathways including insulin/IGF-1, AMP kinase, and protein kinase A (Fontana et al., 2010; Stanfel et al., 2009). Reducing TOR pathway signaling leads to protection from a wide array of age-related diseases. Rapamycin is a specific inhibitor of TOR expression (Sarbassov et al., 2006; Thoreen et al., 2009) and has been widely investigated in aging studies. Inactivation of the TOR pathway increases mitochondrial number and induces mitochondrial gene expression via translational regulation, leading to enhanced cellular respiration (Hur et al., 2010). A major challenge is how to reduce TOR signaling without producing harmful side effects. Another critical issue is to better understand the cross-talk of TOR with other pathways that influence aging (McCormick et al., 2011). It is especially important to know which pathways interact additively with TOR and which interact synergistically.

Besides sensing nutrient availability, TOR integrates signals from changes in growth factors, energy status and various physiological stresses (Sengupta et al., 2010). Each of these inputs modulates TOR expression, which then relays signals to downstream outputs, adjusting metabolism to the environmental circumstances (Katewa and Kapahi, 2011). TORC1 in yeast is rendered less active by high temperature, hydrogen peroxide, and high salt stress (Kapahi et al., 2010). All organisms investigated have genetic programs to cope with temperature, UV, osmotic, and oxidative stress and induction of these stress response programs can slow aging (Haigis and Yankner, 2010). A variety of signals activate cytoprotective programs, including detoxification, innate immunity, proteostasis, and oxidative stress responses that protect cells from a variety of insults (Shore and Ruvkun, 2013). Activation of these programs often leads to lifespan extension, implicating them as key players in aging processes. The mechanism by which these stress responses modulate lifespan and regulate proportionality of the response is still poorly understood.

Among the most versatile and ubiquitous stress sensors in metazoans is the Jun-N-terminal kinase (JNK) signaling pathway (Biteau et al., 2011). JNK is an evolutionarily conserved stress-activated protein kinase that is induced by a range of stressors like UV radiation, reactive oxygen species, DNA damage, heat, bacterial antigens, and inflammatory cytokines (Karpac et al., 2009). Like TOR, JNK adapts metabolism and growth to prevailing environmental conditions (Karpac and Jasper, 2009). JNK has several nuclear and cytoplasmic targets, mostly transcription factors like FOXO, and elicits a variety of tissue-specific responses (Johnson and Nakamura, 2007; Wang et al., 2005). At least 20 JNK genes are known from mammals and their mis-regulation has been implicated in a wide range of neurodegenerative diseases, diabetes and cancer (Weston and Davis, 2007).

JNK also is a mediator of hormesis, increasing stress resistance, autophagy, damage repair, and apoptosis (Le Bourg, 2009; Lee et al., 2009), and its activation can either positively or negatively influence longevity (Twumasi-Boateng et al., 2012). JNK inhibits IIS signaling by regulating insulin sensitivity. This JNK-IIS antagonism regulates metabolic homeostasis (Wang et al., 2005), but the molecular mechanism is complex and poorly understood. JNK also is thought to interact with the TOR pathway, promoting autophagy and fine tuning responses to nutrient conditions (Lee et al., 2009). However, the nature of the JNK interaction with these pathways and understanding of how it regulates metabolism and promotes longer lifespan is very limited.

Many notable advances in understanding aging have come from studying evolutionary divergent species (Austad, 2009). Sequence analysis has produced a clearer understanding of basal metazoan evolution, demonstrating that bilateria animals are organized into the supraphyla Ecdysozoa and Lophotrochozoa (Dunn et al., 2008; Edgecombe et al., 2011). This understanding of phylogenetic relationships focuses attention on the close relationship between the currently popular ecdysozoan animal models of worms and flies and how they might be quite different from mammals because of gene loss. In contrast, sequence analysis indicates that there has been only a minor gene loss in the lophotrochozoans, making them perhaps better models for understanding mammalian aging (Raible and Arendt, 2004). The genome of brachionid rotifers may have an especially high similarity to human genes (Rhee et al., 2012).

These phylogenetic studies also suggest that a major proportion of animal phylogeny remains unexplored with regard to the aging processes. The Lophotrochozoa contains some major animal phyla like Mollusca, Annelida, Platyhelminthes, and Rotifera, the value of which for aging studies is just now being recognized (Ungvari and Philipp, 2011). Lophotrochozoans have intriguing biological properties that are relevant for aging studies, like the great longevity of molluscs, the regeneration of planarians, and the asexual–sexual reproduction of rotifers. Investigation of exceptional animals holds promise for identifying key aging mechanisms that enable them to live long lives and resist a variety of environmental stressors. Studies of these animals might also reveal novel pathways regulating the aging process. A dual strategy of investigating mechanisms of cellular aging coupled with population dynamics and responses to environmental stressors, renders this group an interesting model for studying aging and the evolution of longevity.

We used rotifers as models for studying how TOR signaling modulates aging and to screen other pathways for interaction with TOR. Rotifers are Lophotrochozoans amenable to life table experiments, with a long history of being useful for aging studies (Snell, in press). Some species respond to dietary restriction (DR), with intermittent fasting (IF) being more effective than continuous DR (Gribble and Mark Welch, 2012). IF extended mean lifespan 50–70%, primarily by delaying death in young age classes rather than by preventing death in middle age or extending maximum lifespan. Since rotifers can reproduce by ameiotic parthenogenesis, they can be propagated easily by cloning. This reproductive feature makes it easy to demonstrate multiple aging outcomes from a single clonal lineage, demonstrating an important role of epigenetic programming in the observed patterns of aging (Skold and Obst, 2011). Rotifer lifespan also can be extended by dietary supplements of particular combinations of antioxidants, suggesting additive modes of action (Snell et al., 2012). Exposure to most antioxidants had no effect on rotifer lifespan, only about 12% of those tested produced life extension. Rotifers provided a test system for facile life table analysis that enabled searching through large portions of parameter space to find particular combinations of antioxidants capable of life extension.

In this paper, we screened a variety of signaling pathway inhibitors for their ability to extend lifespan in rotifers without large effects on reproduction. We identified TOR and JNK genes as modulators of important signaling pathways that affect rotifer lifespan. RNAi was used to confirm the specific role of these genes in lifespan regulation. We describe an additive interaction on lifespan of inhibiting both TOR and JNK pathways simultaneously. Treatments that produced lifespan extension also conferred resistance to environmental stressors like starvation, UV, osmotic, and oxidative stress. We observed that treatment of rotifers with rapamycin or JNK inhibitor improved mitochondria activity and reduced lysosomal activity.

2. Methods

2.1. Rotifer cultures

Brachionus manjavacas (Fontaneto et al., 2007), originally called *Brachionus plicatilis* Russian strain and collected from the Azov Sea region in Russia, was used for these experiments. This species has been propagated continuously in the lab since 1983, with periodic resting egg production, collection, and storage.

To obtain animals for experiments, resting eggs were hatched in 25 ml of 15 ppt artificial seawater (ASW, Instant Ocean), under constant fluorescent illumination (2000 lx) at 25 °C. Hatching began after 18–20 h and the hatchlings were fed *Tetraselmis suecica*

cultured in F medium (Guillard, 1983) in a 560 ml chemostat with 1/4 daily medium replacement under constant fluorescent illumination (2000 lx) at 25 °C. Rotifers were fed *T. suecica* in ASW containing 20 μM 5-fluoro-2-deoxyuridine (FDU) to prevent hatching of their amictic eggs and to make the life table experiments easier to perform (Snell et al., 2012).

2.2. Metabolic pathway inhibitors tested for rotifer life extension

There are several commercially available chemical inhibitors that affect the metabolic pathways that are thought to regulate aging. We tested 12 in life table experiments and they are listed in Table 1 along with their source, target, and exposure concentration. Exposure concentrations were determined empirically from reproductive range finding tests where rotifers were exposed to concentrations of 0.1–20 μM and the highest concentration was determined where there was no reproductive inhibition compared to controls. (See Tables 2 and 3.)

2.3. RNAi of TOR and JNK pathway genes

Sequences for the TOR and JNK pathway genes were derived from the *B. manjavacas* Transcriptome Shotgun Assembly project. This has been

Table 1
Metabolic inhibitors tested for ability to extend rotifer lifespan. Doses of each inhibitor were determined from preliminary range finding tests that estimated the maximum dose possible while avoiding reproductive toxicity. Percent inhibition was not measured.

Inhibitor	Source	Target	Exposure (μM)
JNK SP600125	Sigma-Aldrich	SP600125 inhibits the phosphorylation of c-Jun N-terminal kinase (JNK), a stress-activated serine threonine protein kinase that phosphorylates c-Jun, a component of the transcription factor activator protein-1 (AP-1). AP-1 regulates the transcription of numerous genes including cytokines, growth factors, immunoglobulins, inflammatory enzymes, and matrix metalloproteinases (Bennett et al., 2001). SP600125 also blocks cell activation and differentiation and the expression of inflammatory genes COX-2, IL-2, IL-10, IFN-γ, and TNF-α.	1
WYE-354 CD0270	Chemdea	WYE inhibits TOR kinase and shows high selectivity over various PI3Ks and other protein kinases. In vitro, WYE exhibits significant anti-proliferative activity against a panel of tumor cell lines and activates cell cycle progression, apoptosis, while inhibiting protein synthesis and cell size increase (Yu et al., 2010).	10
KU-0063794 CD0274	Chemdea	KU exhibits high specificity for mTOR and is inactive against PI3Ks and other kinases. KU, but not rapamycin, blocks S6K1 activity by phosphorylating the hydrophobic motif and subsequently the T-loop. KU inhibits the phosphorylation of mTORC1 in a dose-dependent manner. KU also induces a dose-dependent inhibition of Akt activity and induces a G1 cell cycle arrest more significantly than rapamycin (García-Martínez et al., 2009).	10
Pp242 CD0258	Chemdea	PP242 exhibits strong selectivity for mTOR over other PI3K family kinases. PP242 displays some inhibitory activity against Ret, PKCα, PKCβ, and JAK2, while exhibiting remarkable selectivity against 215 other protein kinases. PP242 is a more effective mTORC1 inhibitor than rapamycin, but unlike rapamycin, PP242 inhibits both mTORC1 and mTORC2. PP242 also inhibits the phosphorylation of Akt and its downstream target S6 dose-dependently (Apsel et al., 2008).	10
Rapamycin	Sigma-Aldrich	Rapamycin inhibits the TOR complex 1 (TORC1), a serine/threonine kinase composed of TOR and its associated proteins. It is the central element of the TOR signaling network, monitoring a diverse set of intra- and extracellular signals. It controls cell size, proliferation, and lifespan through a variety of downstream pathways. Rapamycin treatment induces G1/S cell cycle arrest, autophagy, and strong inhibition of translation initiation (Takeuchi et al., 2005).	1
HSP90 17-AAG	Selleck	HSP90 inhibitor causes the degradation of HER2, HER3, Akt proteins, and androgen receptor (AR), leading to G1 growth arrest and apoptosis. Administration of 17-AAG (~50 mg/kg) causes >50% decline in AR, HER2, HER3, and Akt expression (Kamal et al., 2003).	1
p38 BIRB 796	Selleck	BIRB 796 represents one of the most potent and slowest dissociating inhibitors against human p38 MAP kinase known. BIRB 796 potently inhibits c-Raf-1 and Jnk2α2 with IC50 of 1.4 and 0.1 nM, respectively. BIRB796 also inhibits the activity and the activation of SAPK3/p38γ at a higher concentration than it does in p38α. BIRB796 blocks the stress-induced phosphorylation of the scaffold protein SAP97, which is a physiological substrate of SAPK3/p38γ. Moreover, the binding of BIRB796 to the p38 MAPKs or JNK1/2 is impairing their phosphorylation by the upstream kinase MKK6 or MKK4 rather than enhancing their dephosphorylation.	1
Wnt XAV-939	Selleck	XAV-939 specifically inhibits tankyrase PARP activity. XAV-939 dramatically decreases DNA-PKcs protein levels, confirming the critical role of tankyrase poly-ADP-ribosylation activity in maintaining stability of the DNA-PKcs protein. XAV-939 stimulates beta-catenin degradation by stabilizing axin, the concentration-limiting component of the destruction complex. XAV-939 stabilizes axin by blocking the poly-ADP-ribosylating enzymes tankyrase 1 and tankyrase 2. XAV-939 deregulates the Wnt/b-catenin pathway which has been implicated in many cancers.	10
IGF-1R GSK1904529A	Selleck	GSK1904529A is a reversible, ATP-competitive inhibitor and has enzyme-inhibitor binding values against IGF-1R and IR with K_i of 1.6 nM and 1.3 nM, respectively. GSK1904529A potently inhibits the ligand-induced phosphorylation of IGF-1R and IR at concentrations above 0.01 μM, followed by blocking downstream signaling (AKT, IRS-1, and ERK). GSK1904529A potently inhibits NIH-3T3/LISN, TC-71, SK-N-MC, SK-ES RD-ES cells with IC50 of 60 nM, 35 nM, 43 nM, 61 nM and 62 nM, respectively. GSK1904529A induces cell cycle arrest at the G1 phase in cell lines COLO 205, MCF-7, and NCI-H929, which are sensitive to GK1904529A.	10
AMPK p5499	Sigma-Aldrich	Dorsomorphin is a selective inhibitor of bone morphogenetic protein (BMP) signaling. It has been found to inhibit BMP signals required for embryogenesis and promoted significant neural differentiation from human pluripotent stem cell (hPSC) lines (Dolez et al., 2011). Dorsomorphin also acts as a potent, selective, reversible, and ATP-competitive inhibitor of AMPK (AMP-activated protein kinase); Ki = 109 nM in the presence of 5 μM ATP and the absence of AMP.	10
PI3K AS-605240	Selleck	AS-605240 is an ATP-competitive PI3Kγ inhibitor, with Ki values of 7.8 nM. AS-605240 is isoform-selective, for AS-605240 also inhibits PI3Kα, β, and δ, with IC50 of 60, 270, and 300 nM, respectively. At SC-CA1 synapses in mice, AS-605240 (100 nM) eliminates NMDAR LTD, without affecting mGluR LTD, depotentiation, and LTP.	1
p53 pifithrin-α	Stemgent	Pifithrin-α inhibits p53-dependent transactivation of p53-responsive genes in ConA cells. Pifithrin-α also inhibits p53-dependent growth arrest of human diploid fibroblasts in response to DNA damage but has no effect on p53-deficient fibroblasts. Pifithrin-α may modulate the nuclear import or export (or both) of p53. Pifithrin-α also decreases the basal level of p53 DNA-binding activity. Pifithrin-α stabilizes mitochondrial function, suppresses caspase activation and protects cultured hippocampal neurons against death induced by glutamate and amyloid β-peptide. Pifithrin-α also can suppress heat shock and glucocorticoid receptor signaling. Pifithrin-α reduces activation of heat shock transcription factor and increases cell sensitivity to heat.	10

Table 2
List of primer sequences used in PCR of TOR and adipocytokine signaling pathway genes. T7 PCR primers are identical with the addition of the T7 adapter at the 5′-end. The isotig numbers refer to the corresponding sequences in the *Brachionus manjavacas* transcriptome shotgun assembly project, under accession GARS00000000.

Gene	Isotig	Pathway	Forward primer	Reverse primer
JNK1	04620	Adipocytokine	CCAAACGAGCTTACCGAGAG	ACCGTTCCATGAAGTCGTTC
TOR2	01986	TOR Adipocytokine	TTATCCGGCTCTGGCAATAC	CTAACCCAATTTGCCGACTC
AKT14	02154	TOR Adipocytokine	CGAGCCTCCACTTCTCAAAC	GCGTACTCCATCACAAAGCA
AMPK2	01216	TOR	GAGCGGAGGAGAATTGTTTG	CTGGTGCAGGTTTTGGAAAT
rapTOR3	02056	TOR	AAATACCCGGCCAAATAAGC	AGCTTGAGCACGTAGGGAAA
RHEB2	08779	TOR	GTATTGGGCAGTGGAGGTGT	TTTTGTTTCCCTTGGCATTC
PI3K	02289	TOR	TGCCACTGGGGTAAAAGAAG	CTGAGCTTGTTCATCGGTCA
GBL2	05985	TOR	CCATCGGCTTTAAGGACAAA	CTGTGCCATCTGCTGAAGAA
TSC22	03082	TOR	TGtACCATtCGTCGTTcGAG	AAGCGCTTTTGAGCATTTGT
ID	02322	Adipocytokine	TCCGGATGAAAAGATCCAAG	TCGAACCTTTGCGGATTTAC
MapKL	04774	Adipocytokine	CACGACCATACCGGATTTTT	CGGACATTCGCAAATAGGTT
TNFr	06427	Adipocytokine	TCCCTCGATTAGGGAGAGTG	TCGAGGGCGAAAATAATTCA
TRAF2	05364	Adipocytokine	CACAGCATGAACGCTGAAGT	TCCCAAAATTTCCTTTGCAG

deposited at DDBJ/EMBL/GenBank under the accession GARS00000000. The version described in this paper is the first version, GARS01000000. Genes were selected from various points in the TOR signaling pathway, PI3K, AKT1, AKT2, GBL, TOR, TSC2, Raptor, AMPK, and Rheb. Primer sets for each gene were developed that amplified a 500-bp band for RNA interference. Primer sets that generated a single, strong band were re-ordered with a T7-motif (TAATACGACTCACTATAGG) on the 5′-end. The T7 primers were used to produce PCR products of the gene of interest using Go-Taq DNA polymerase (Promega) with 1 mM MgCl2, 100 mM dNTP (Promega), 10× buffer, 0.5 μM forward primer, 0.5 μM reverse primer, 1 μ Taq (5 μ/μl) and ~400 ng DNA. These reactions were then transcribed into dsRNA using T7 RNA polymerase (Promega) with 10 mM DTT, 5× Promega buffer, 100 μM NTP (Invitrogen), added to the whole 10 μl T7 PCR reaction. The transcription was incubated at 37 °C for 4 h. The dsRNA was precipitated by adding 5 μl sodium acetate and 100 μl 95% ethanol and incubated at 4 °C for 18–24 h. The dsRNA was pelleted by centrifugation at 14.8 thousand rpm for 15 min. The pellet was then washed in 500 μl 70% ethanol and re-pelleted at 14.8 thousand rpm for 5 min. All ethanols were removed and the pellet dried.

2.4. Estimating dsRNA

The dsRNA pellet was re-suspended in 10 μl of water. Two samples (1× and 4×) of the dsRNA were run on a 2% agarose gel and the amount of dsRNA was estimated in relation to 5 μl 100 bp DNA ladder (Invitrogen). ImageJ was used to estimate the relative intensity of the 500 bp band of the ladder as well as the relative intensity of 1× or 4× sample of the dsRNA. The background intensities were subtracted with the same area as the bands. The amount of dsRNA in the sample was estimated relative to the pixel intensity of the 500 ng of DNA in the ladder for the 500 bp band.

2.5. Decapsulation and transfections

Diapausing rotifer embryos were decapsulated using the technique of Snell et al. (2011). Decapsulated resting eggs were kept overnight at 4 °C before transfections to thoroughly rehydrate and ensure synchronous hatching. Transfections were performed using 1 μl Fugene 6 (Promega) in 44 μl phosphate buffered saline. For the resting egg and the subsequent feeding transfections, approximately 1000 ng dsRNA was resuspended in 5 μl water. Repetitive feeding transfections were performed on hatchling *B. manjavacas* (day 0), 2 day old and 4 day old females; all were exposed to the transfection solution for 4 h before transfer to growth media. This triple exposure to the RNAi transfection solution prolonged the knockdown effect longer than a single exposure.

2.6. RNA isolation and quantitative PCR

For rapamycin, JNK inhibitor and RNAi experiments, five day old rotifers were collected and placed individually in 20 μl of RNAlater (Qiagen) and stored at −80 °C. RNA isolation was performed using RNeasy MinElute Cleanup Kit (Qiagen), eluting in 14 μl water. RT-qPCR primers were designed within the 500 bp region originally targeted by RNAi, and were designed to amplify a 200 bp fragment. To verify that the qPCR primers amplified only a single product, the primers were used to amplify a PCR product of the gene of interest from *B. manjavacas* cDNA, according to the same methods that the T7 PCR products were obtained (see above), and the presence of a single band was confirmed on a 2% agarose gel under UV exposure. The PCR products were confirmed by sequencing by Eurofins MWG Operon (Huntsville, AL). RT-qPCR was conducted using EXPRESS One-Step SuperScript® qRT-PCR Universal with SYBR green for specific DNA detection (Invitrogen) and gene specific primers on a Mastercycler Realplex 2 (Eppendorf). Master mixes were prepared according to kit protocol and contained 5 μl of RNA template, diluted 1:10. Each RT-qPCR plate contained 8 biological replicates per treatment, and each reaction was performed in triplicate. Cycling parameters were 50 °C for 2 min, 95 °C for 5 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Prior to melt curve analysis, PCR products were heated to 95 °C for 3 s, then cooled to 60 °C for 15 s. Data was collected as threshold cycle (C_T) values. Expression levels of target genes were obtained by normalizing to internal reference genes, and fold

Table 3
List of primer sequences used in RT-qPCR to assess TOR and JNK gene expression normalized to actin, a reference gene involved in structural integrity (Snell et al., 2011). Target gene primer sequences were designed to amplify an approximately 200 bp region of the TOR or JNK genes.

Gene	Isotig	Pathway	Forward primer	Reverse primer
qJNK1	04620	Adipocytokine	GGCCAACGCTAACTTTTCAA	ACGCAGCATTTCACCAAATA
qTOR2	01986	TOR adipocytokine	GTGGCAAATAGCGCTTGAAT	AGCCAAGAAGGAGACAAGCA
qActin	0XXXX		GCATCCACGAGACCACCTAT	TAGGATCGAACCACCAATCC

changes in the relative gene expression were calculated according to the ΔΔCt method (Livak and Schmittgen, 2001). Actin was used as a reference gene for all treatments. Primer efficiencies were verified with standard quantification curves. Amplification efficiencies of the primers, which were approximately 80–100%, were determined from the slope of the calibration curve. Statistics were performed on ΔCT values (Yuan et al., 2006), using t-tests to reveal significant differences in expression of treated rotifers relative to controls.

2.7. Experimental design and treatments

The effects of the chemical inhibitors on rotifers were tested first in a 3-day reproductive test (Snell et al., 2012) to determine the highest concentration where there was no significant depression of reproduction. For all the inhibitors this was either 1 or 10 μM. Full cohort life tables were then conducted at 22 °C with 120 female rotifers per treatment, and cultured 5 individuals per well in 1 ml in 24-well plates. Repeated ANOVAs to detect well-effects failed to find any under our experimental conditions, so we treated each of the 120 rotifers in a treatment as an independent estimate of the lifespan. Data is reported as mean, median, and maximum lifespan (age of 95% mortality). Each test well received 1 ml of 15 ppt ASW containing 6×10^5 *T. suecica* cells/ml at the beginning of the experiment and all surviving rotifers were transferred to fresh medium on day 8. All wells were checked daily and rotifer survival was recorded until all animals died, usually about 21 days. Each well also contained 20 μM FDU which allows amictic eggs to be produced, but prevents their hatching. This eliminated the requirement of removing offspring from each well daily, simplifying the execution of the life table experiments. Because both rapamycin and JNK inhibitor have limited water solubility, they were dissolved first in DMSO and then diluted with ASW to the test concentrations. The highest DMSO concentration that rotifers were ever exposed to was 0.3%, so this was added to our ASW controls. We never observed any effects on rotifer survival or reproduction with this concentration of DMSO.

To observe reproductive effects of the inhibitors, rotifers were placed in 24-well plates with 2×10^5 *T. suecica* cells/ml and no FDU. Each treatment consisted of 24 wells with 1 animal in each well. The number of offspring produced by each parthenogenetic mother was recorded, and all offspring were removed daily until all original maternal females were dead. Rotifers were transferred to fresh medium on day 7. Since no eggs were hatched in 1 μM JNK inhibitor treatments, the number of eggs dropped was recorded as the number of offspring produced.

The ability of RNAi to knock down the expression of selected genes was initially tested using a 10 day survival screen. This enabled us to test many treatments quickly to determine which treatments to examine in more detail in life table experiments. For a 10 day screen, rotifers were placed 7 animals per well in 24-well plates and incubated at 25 °C in 1 ml 15 ppt ASW, containing 6×10^5 *T. suecica* cells/ml and 20 μM FDU. Each treatment consisted of 4 wells and a total of 28 animals. These tests were scored on days 1, 7, and 10 for survival and compared to the TOR scramble transfection control. Rotifers used for qPCR were transfected as resting eggs, hatchlings, and as 2 and 4 day old animals. After the hatchling transfection, animals were fed 6×10^5 *T. suecica* cells/ml in 15 ppt ASW and were cultured at 22 °C before collection on day 5 for RNA extraction and qPCR.

2.8. TOR scramble

As a control for mortality caused by RNAi transfections and exposure to dsRNA, the rotifer TOR gene was scrambled using a sequence scramble program (http://users.umassmed.edu/ian.york/Scramble.shtml). The resulting sequence was produced by the company IDT and was used in conjunction with T7 primers to produce a ~300-bp sequence of dsRNA for RNAi. This scramble sequence does not appear anywhere in the *B. manjavacas* known genome and was used as the control in all RNAi transfection experiments and qPCR. The scramble sequence is: TTAGACTATAAATCCTTATAAAAAGTCCCATTGGTCTAAGTTCCATCCTTT CAGGGAAAGTACCTTAACCACGGCTTGTATTTTACGTGCTGTGTGTTTAA GAGATCAACGAATCGGGATGTTTACTTTGCGGGATACATAGTTATAAGCT GTTGCAAAGTATAAGCAGCTAACCCGTTAAATATGAACGTGGAAGATTGC AACTTTGCGAGTACATGAtATCTTTTGTAATGTCCCAGATCAGGGAAACGC-TTTGGAATAAGTCCTAAAATCGTTCAGTCATGTAGAACGCTTAGTATTTTCT TTAACACGTTTACAACTAAAGGCTA. Forward and reverse PCR primers are underlined and highlighted in yellow.

2.9. Stressor challenge experiments

Rotifers were exposed to either 1 μM rapamycin, 1 μM JNK inhibitor, or 0.5 μM each of rapamycin and JNK inhibitor for four days, and fed 6×10^5 *T. suecica* cells/ml with 20 μM FDU at 25 °C. Then these animals were washed of algae, exposed to various stressors, and transferred to fresh ASW without algae for 72 h recovery when survival was recorded. The stressors included starvation by denying food for 72 h, exposure to UV for 20 min, continuous exposure to 0.1 μM juglone for 72 h, osmotic shock by transferring from 15 ppt to 60 ppt for 2 h and return to 15 ppt, or heat shock by exposure to 40 °C for 1 h, then return to 22 °C for 72 h. The UV exposure was to 20 min of UV–B radiation, 25 cm from an 8 W source (UVP, model UVM-28 EI) with an intensity peak at 302 nm. Rotifers were exposed in 5 ml ASW in a 55 mm diameter petri dish so that they received a dose of about 130 J m^{-2} (Preston et al., 1999). The 24 h LD50 for UVB exposure for a closely related marine *Brachionus* species is 24,600 J m^{-2} (J.I. Kim et al., 2011; R.O. Kim et al., 2011). Exposure to juglone causes oxidative stress (Snell et al., 2012). These stressor exposures were chosen because they were strong enough to produce considerable mortality after 72 h, but not 100% mortality. After each stressor challenge, rotifer survival was assessed after 72 h and reported as proportion surviving from a cohort of 120 animals.

2.10. Estimation of mitochondrial activity using MitoTracker and lysosome activity using LysoTracker

Rotifers were incubated in 24-well plates with 6×10^5 *T. suecica* cells/ml and 20 μM FDU at 22 °C for four days. Treatments were 1 μM JNK Inhibitor, 1 μM rapamycin, or a combination of 0.5 μM JNK inhibitor and 0.5 μM rapamycin, plus a 0.3% DMSO control. Before staining, animals were rinsed in fresh ASW for 1 h to clear their guts and eliminate auto-fluorescent algae. Animals were then incubated with 5 μM MitoTracker® Red (Invitrogen) for 30 min in the dark. The rotifers were then rinsed with ASW, anesthetized with 1 ml club soda, fixed with 20 μl 20% formalin, and rinsed with ASW again. To ensure that there was no nonspecific binding of the fluorescent compound, 15 animals from each treatment were anesthetized, fixed with formalin, and incubated at room temperature for 4 h before staining and imaging. The same protocol was used for LysoTracker® Red (Invitrogen), with the exception that the concentration of LysoTracker® Red was 1 μM. To produce an age series of lysosome activity, animals were stained and images taken after 0, 1, 2, 4, 6, 8, 10, and 12 days treatment. Images were taken at 200× magnification with an Alexa 486 nm filter using a Zeiss Imager.Z1 microscope. For MitoTracker® Red, camera exposure was 5 ms, and for LysoTracker® Red, exposure was 20 ms. Average pixel intensity of each animal was analyzed using ImageJ by selecting the entire animal as the region of interest, and then subtracting the pixel intensity of the background. Fifteen animals were imaged for each treatment, and the mean pixel intensity was calculated for each treatment.

2.11. Estimation of rotifer swimming speed

We tested the effect of 1 μM rapamycin and 1 μM JNK inhibitor exposure on the swimming speed of 10 day old females. Other treatments included a control and a combined treatment of 0.5 μM rapamycin

+ 0.5 μM JNK inhibitor. Several dozen rotifer hatchlings were maintained for 10 days in 5 ml ASW containing 6×10^5 *T. suecica* cells/ml and 20 μM FDU at 25 °C. At age 10 days, the swimming behavior of 15 randomly chosen females from each treatment was recorded at 22 °C at 15× magnification using a PixeLink camera attached to a stereomicroscope. One rotifer was placed on a painted slide with 10 dots in about 15 μl of ASW. Two layers of tape on each end of the slide provided support for a cover slip and space for the rotifer to swim. A 30 second AVI video at 50 frames/s was recorded for each rotifer, transferred to VirtualDub (Lee, A., 2008, v.1.7.8, www.virtualdub.org) to convert the entire video to image portable network graphic image sequences, then imported in ImageJ for analysis. Image stack merger plus (Péan, S., http://www.samuelpean.com/image-stack-merger-plus/, a plugin for ImageJ) was used to subtract rotifers from the image sequences and to create a clean background reference image. This reference image was used to subtract the background from the complete video. The video threshold was adjusted so that the rotifer was black on a white background, and all visible artifacts were removed. The minimum and maximum pixel sizes of the rotifer was entered into the Mtrack2 plugin (Stuurman, N., 2003, http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html), which outputted x and y pixel coordinates per frame, to give a pixel per frame velocity. Average velocity per rotifer video was calculated, and converted to mm per second using an average of 102.3 pixels/mm, and a frame-rate of 50 frames/s.

2.12. Statistical analysis

Mean lifespan was compared by one-way analysis of variance followed by Dunnett's test to compare treatments to control using the statistical package JMP 8 (SAS Institute). Survival curves were compared using the JMP 8 reliability and survival analysis, with a Wilcoxon's test calculated to compare survival curves of control and treatments. Stress tests, MitoTracker and LysoTracker staining intensities, and female swimming speed were compared by ANOVA with Dunnett's test to compare treatments to control.

3. Results

We screened 12 commercially available chemical inhibitors of a variety metabolic pathways for their effect on the survival of *B. manjavacas* (Fig. 1a & b). Only three significantly extended mean lifespan compared to the control: 1 μM rapamycin and JNK inhibitor, and 10 μM KU. The first two extended life by 19% and the latter by 11%. A Wilcoxon test of the Kaplan–Meier survival curves indicated that all three of these treatments significantly extended lifespan over the control. The inhibitor of AMPK at 10 μM significantly shortened rotifer lifespan by 51%, but exposure to 0.5 μM had no effect on lifespan. The targets of these inhibitors and their metabolic effects are described in Table 1.

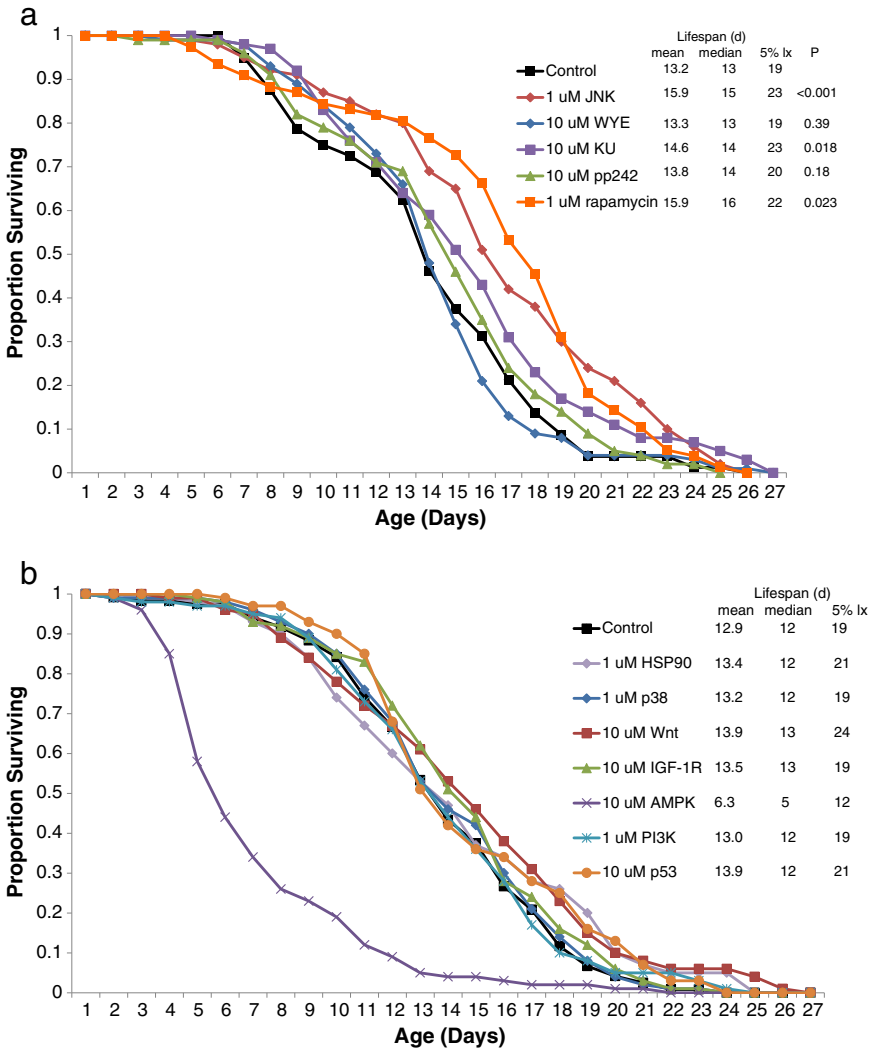

Fig. 1. a) Survival curves for *B. manjavacas* exposed to TOR pathway inhibitors. b) Survival curves for *B. manjavacas* exposed to other pathway inhibitors. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. Inhibitors are described in Table 1. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control.

A second life table experiment with rapamycin was performed, this one without FDU so that we could examine rapamycin effects on reproduction (Fig. 2a & b). Rotifers treated with 1 μM rapamycin had 35% longer mean lifespan, 29% longer median lifespan, and 53% longer maximum lifespan than controls. Rapamycin exposure had no significant effect on total lifetime reproduction of females, but peak reproduction was shifted from day 4 in controls to day 6 (Fig. 2b). The length of the reproductive lifespan was extended in the rapamycin treatment to 11 days from 6 days in controls. The length of the pre-reproductive lifespan was similar in control vs. rapamycin treated animals, but the post-reproductive period was significantly shorter in the latter.

A second life table experiment with 1 μM JNK inhibitor, but without FDU extended mean and median lifespan by 35% and maximum lifespan by 37% (Fig. 3a). JNK inhibitor significantly reduced total lifetime reproduction by 77% compared to the average 21.8 offspring produced by control females (Fig. 3b). In the rapamycin reproductive experiment, fully developed, swimming hatchlings were recorded as offspring. In contrast, the JNK inhibitor treatment allowed eggs to form normally, but none completed development and hatched into rotifers. So the "offspring" in this latter experiment were recorded as unhatched eggs. If we reduced the JNK inhibitor concentration to 0.25 μM, no suppression of egg hatching was observed and reproduction was indistinguishable from controls.

A third life table experiment was performed to examine whether simultaneous exposure to rapamycin and JNK inhibitor produced interactive effects. In this experiment we observed a more modest life extension with JNK inhibitor (19.5%) or rapamycin (15.9%) treatments alone (Fig. 4). However, combined treatment with 0.5 μM rapamycin and JNK inhibitor extended rotifer mean lifespan by 33%. This apparently additive interaction between rapamycin and JNK inhibitor motivated us to explore this effect further.

Additive interaction between rapamycin and JNK inhibitor treatments was confirmed in stressor challenge experiments (Fig. 5). In these experiments, populations of 4 day old experimental animals were exposed to a stressor challenge like starvation, UV, oxidative, osmotic or heat stress and then survival was assessed after 72 h. In the starvation challenge, in untreated controls after 72 h of starvation, only 20% of the original 120 test animals remained alive. If rotifers were exposed to either 1 μM rapamycin or JNK inhibitor for four days prior to starvation, survival increased to over 50%. If rotifers were exposed to both 0.5 μM rapamycin and JNK inhibitor simultaneously, survival increased to 83%. A similar additive effect of rapamycin and JNK inhibitor treatment was observed for the UV and osmotic challenges,

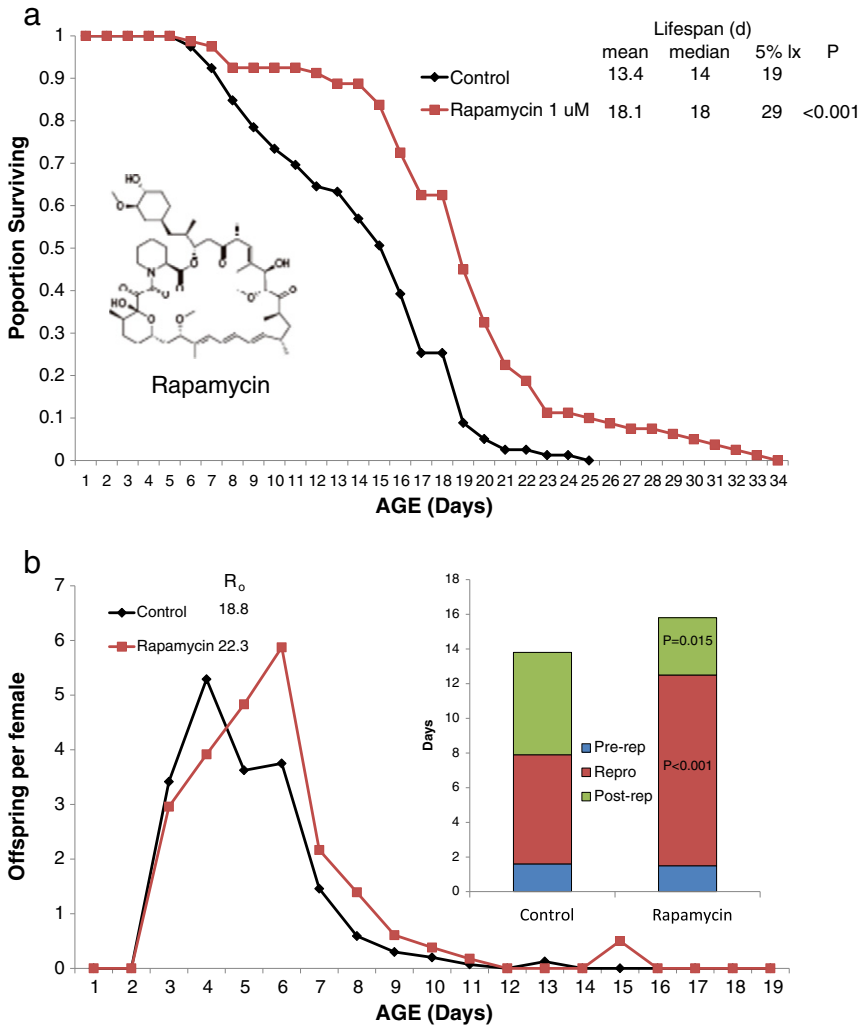

Fig. 2. a) Survival curves for *B. manjavacas* continuously exposed to 1 μM rapamycin. b) Reproduction curves for *B. manjavacas* exposed to 1 μM rapamycin. Proportion surviving represents the fraction of an initial cohort of 48 rotifers surviving to the indicated age. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control. Offspring per female refers to the average number of offspring produced daily by a cohort of 48 females. R_o is the mean total offspring produced by a female over her lifetime. Pre-rep is the number of days in the mean lifespan spent in the pre-reproductive stage, repro is the reproductive stage, and post-rep is number of days spent post- reproductive. P values imbedded in the columns refer to the probability that the reproductive stages of the rapamycin treated females differ significantly from controls.

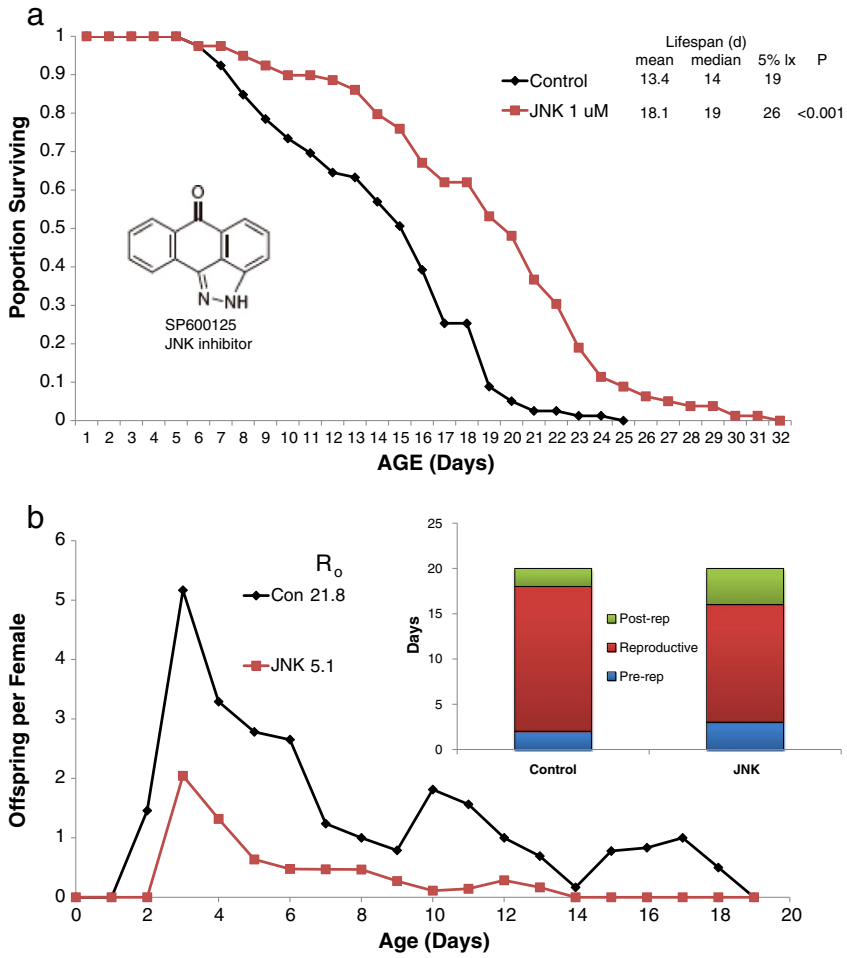

Fig. 3. a) Survival curves for *B. manjavacas* continuously exposed to 1 μM JNK inhibitor (SP600124). b) Reproduction curves for *B. manjavacas* exposed to 1 μM JNK inhibitor. Proportion surviving represents the fraction of an initial cohort of 48 rotifers surviving to the indicated age. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control. Offspring per female refers to the average daily reproduction by a cohort of 48 females. R_o is the mean total reproduction of a female over her lifetime, which is offspring for the control, but only eggs for the JNK treatment. Exposure to JNK inhibitor allows eggs to be produced but prevents their hatching into offspring. Pre-rep is the number of days in the mean lifespan spent in the pre-reproductive stage, repro is the reproductive stage, and post-rep is number of days spent post- reproductive.

but not for the oxidative and heat stressors. Clearly, inhibiting TOR or JNK pathways enables rotifers to better resist some kinds of stress, but the simultaneous inhibition of both of these pathways produces the greatest stressor resistance.

We investigated whether rapamycin or JNK inhibitor treatment protected mitochondria from diminished activity with aging (Fig. 6). MitoTracker is a nonfluorescent compound that is oxidized to a highly fluorescent compound inside mitochondria and a thiol conjugation

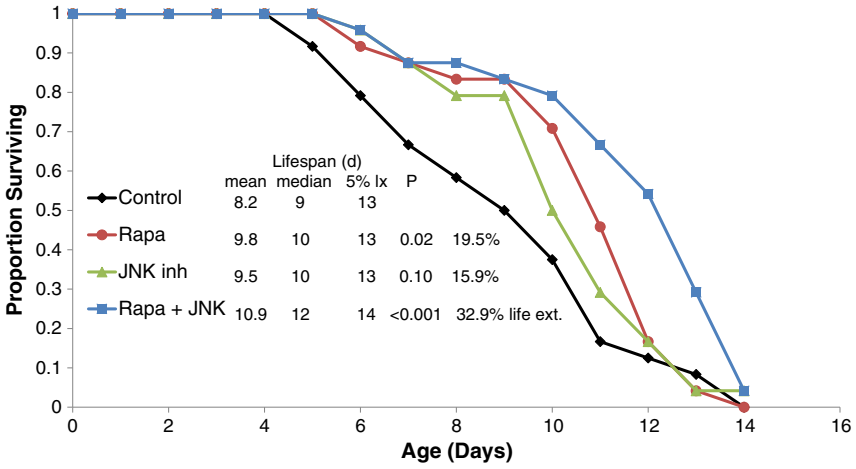

Fig. 4. Survival curves for *B. manjavacas* demonstrating additive effects of exposure to both TOR and JNK pathway inhibitors. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control. The concentration in each of the rapamycin and JNK inhibitor treatments was 1 μM, and 0.5 μM in each rapamycin and JNK inhibitor in the combined treatment.

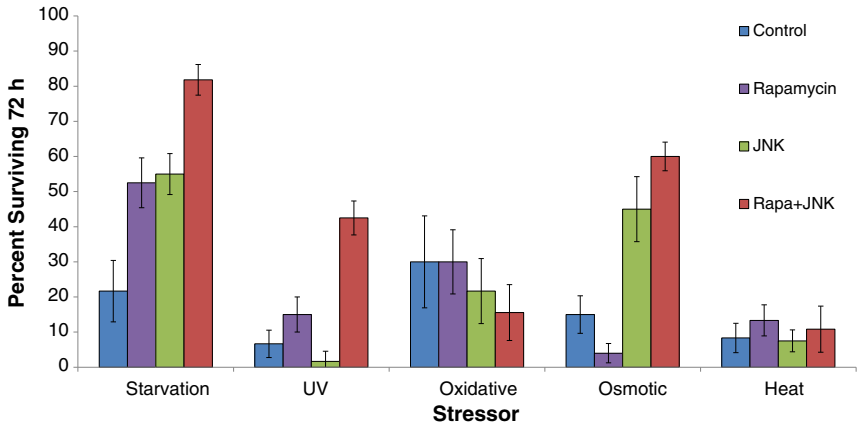

Fig. 5. Effect of stressor challenges on *B. manjavacas* survival. Test rotifers in the rapamycin and JNK inhibitor treatments were exposed to 1 μM for four days before the application of each stressor. The combined rapamycin + JNK treatment was exposed to 0.5 μM of each compound. The starvation test denied rotifers food for 72 h. The UV, osmotic and heat stress tests applied stressors for 20 min, 2 or 1 h, respectively, and the oxidative stress test exposed rotifers to 0.1 μM juglone continuously. Proportion surviving is the fraction of a cohort of 120 rotifers surviving 72 h after stressor exposure. Variance in each treatment is indicated by the standard error bars on top of each column.

sequesters it there for quantitative analysis using epifluorescence microscopy. In untreated controls, average pixel intensity was 7 fluorescence units in 4-day old live rotifers, but only 2 in similar age rotifers that had been dead for 4 h. This demonstrates the need for active mitochondria to metabolize MitoTracker and produce fluorescence. When rotifers were treated with 1 μM rapamycin for 4 days and then exposed to MitoTracker, there was no significant difference in fluorescence intensity from the control. In contrast, treatment with 1 μM JNK inhibitor for four days and then staining with MitoTracker produced more than a two-fold increase in fluorescence, suggesting that rotifers treated with JNK inhibitor had more active mitochondria at age 4 days than control animals. Combined inhibition of TOR and JNK pathways did not preserve mitochondrial activity more than untreated controls. All of the effects were due to JNK inhibition.

LysoTracker was used to estimate rotifer lysosome activity (Fig. 7). Lysosome activity peaked in 6 day old females at levels approximately 3-fold higher than newborns. Treatment for 1 day with 1 μM rapamycin or JNK inhibitor produced significantly less lysosomal activity than the controls (Fig. 8). The combined treatment of 0.5 μM rapamycin and JNK inhibitor produced no further reduction in lysosome activity. A similar effect was observed in 8 day old females, but the magnitude of reduction in lysosome activity was a bit less.

A similar experimental design was employed to investigate rotifer swimming speed as an endpoint (Fig. 9). Swimming speed of 10 day old control rotifers was 1.39 mm/s or 3–4 body lengths/s. When rotifers were exposed to 1 μM rapamycin or JNK inhibitor for 10 days, there was no significant change in their swimming speed compared to controls. However, when simultaneously exposed to 0.5 μM rapamycin and JNK

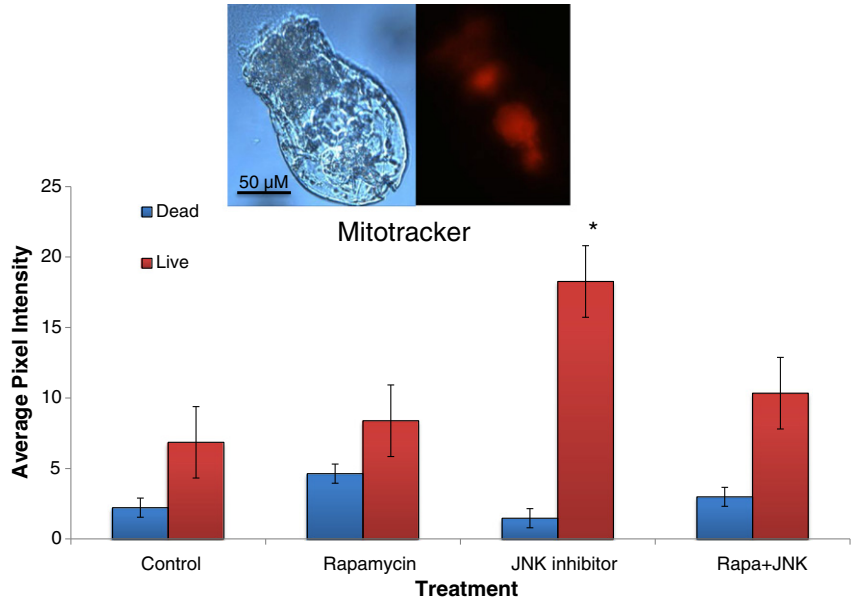

Fig. 6. Estimating differences in mitochondria activity using MitoTracker. Rotifers were exposed to 1 μM rapamycin or JNK inhibitor, or 0.5 μM rapamycin + JNK inhibitor for two days and then stained with MitoTracker. Mitochondria activity in live rotifers is compared to rotifers that had been dead for 4 h. The average pixel intensity refers to the mean epiflourescence observed in treated rotifers compared to untreated controls. Asterisks indicate significant differences from the control by ANOVA and Dunnett's test. Vertical lines indicate standard error.

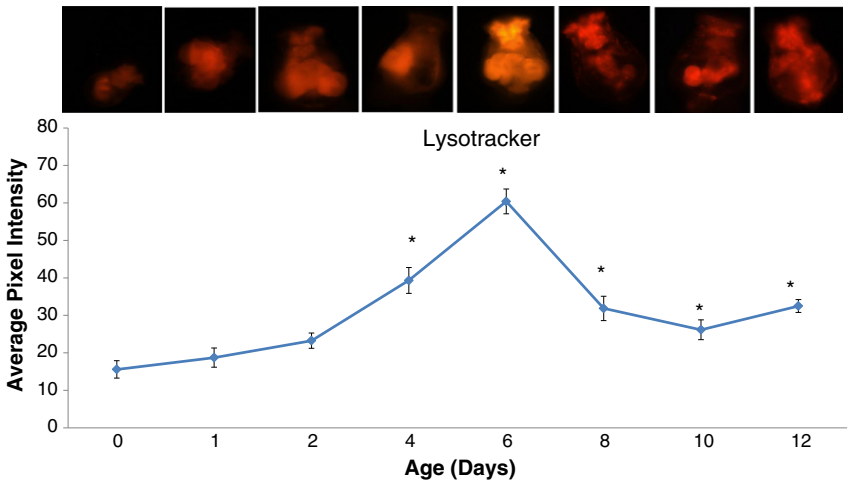

Fig. 7. Estimating changes in rotifer lysosome activity with age using LysoTracker. Rotifers were maintained in ASW at 22 °C with food until the appropriate age and then stained with LysoTracker. The average pixel intensity refers to the mean epiflourescence observed in treated rotifers compared to untreated controls. Asterisks indicate significant differences from the control by ANOVA and Dunnett's test. Vertical lines indicate standard error.

inhibitor, 10 day old rotifers were swimming on average 28% faster than the controls.

We confirmed the important role of TOR gene expression inhibition in rotifer lifespan extension by RNAi. We developed three sets of primers from different parts of the TOR gene (TOR1, 2, 7) and used these to make dsRNA in vitro that was transfected into rotifers to elicit an RNAi response. The approximately 500 bp TOR2 fragment was particularly effective for rotifer life extension, producing 27% longer mean lifespan than controls (Fig. 10). Similarly, median lifespan was 30% and maximum lifespan 17% longer, in the TOR2 RNAi treatment. Likewise RNAi inhibition of JNK gene expression extended rotifer lifespan (Fig. 11). Transfection of rotifers with an approximately 1000 bp dsRNA fragment from either JNK1 or 2 primers significantly extended mean lifespan up to 51%, median lifespan 56% and maximum lifespan 38%. As a result of this success in extending rotifer lifespan by RNAi treatment, we screened several other TOR pathway genes for their life extending effects. RNAi knockdowns of the genes RHEB, rapTOR, PI3K, GBL, AMPK, and TSC failed to extend rotifer lifespan. Besides TOR and JNK genes, RNAi knockdown of only the AKT gene produced significant rotifer life extension (Fig. 12), with a 14% longer mean, 15% longer median and 5% longer maximum lifespan. Simultaneous RNAi knockdown of TOR and JNK genes did not produce greater life extension.

We estimated the percent knockdown by our RNAi treatments using qPCR (Fig. 13). For the JNK gene this was 58%, 69% for TOR, and 65% for AKT. As noted above, the knockdowns of each of these genes produced significant rotifer lifespan extension. RNAi knockdowns of AMPK and rapTOR genes were similarly effective (75% and 48%), but failed to produce rotifer life extension.

4. Discussion

This paper illustrates the value of rotifers as models for aging studies by screening a variety of signaling pathway inhibitors for their impact on lifespan. Worm and fly models have the advantage of the availability of many well-characterized mutants to explore aging. However, the ease of performing life table analyses with rotifers is useful for screening large numbers of treatments for life extension. The availability of an extensive molecular tool kit is important for investigating the mechanisms of cellular aging and dissecting metabolic pathways, but some aging problems are best investigated in vivo with whole animal models like rotifers. Rotifers also seem well suited for examining how the inhibition of certain signaling pathways confers resistance to environmental stressors and how inhibition of multiple pathways interacts to produce life extension.

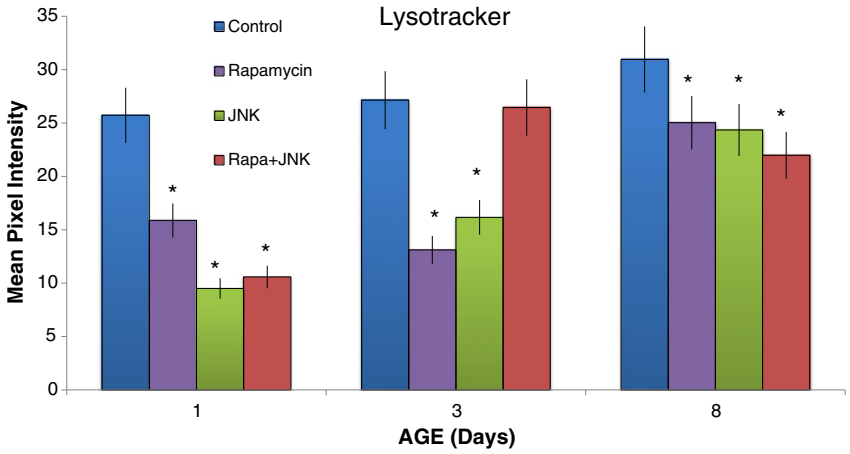

Fig. 8. Estimating lysosome activity in *B. manjavacas* females using LysoTracker. Rotifers were exposed to 1 μM rapamycin or JNK inhibitor, or 0.5 μM rapamycin + JNK inhibitor for 1, 3, or 8 days and then stained with LysoTracker. The average pixel intensity refers to the mean epiflourescence observed in treated rotifers compared to untreated controls. Asterisks indicate significant differences from the control by ANOVA and Dunnett's test. Vertical lines indicate standard error.

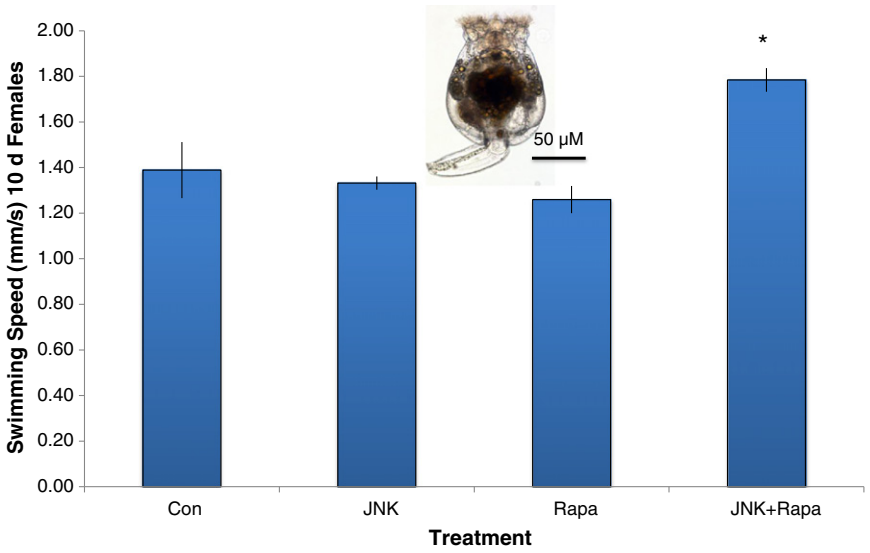

Fig. 9. Estimating differences in swimming speed of 10 d old rotifers exposed to 1 µM rapamycin or JNK inhibitor, or 0.5 µM rapamycin + JNK inhibitor. The average swimming speed (mm/s) of 15 rotifers in each treatment was compared to controls. Asterisks indicate significant differences by ANOVA and Dunnett's test. Vertical lines indicate standard error.

Our screening of signal pathway inhibitors for their ability to extend lifespan in rotifers without large effects on reproduction identified the TOR and JNK pathways as critical modulators of longevity. The role that TOR signaling has in nutrient sensing, stress resistance, and life extension is widely appreciated (Sharp, 2011), but the impact of JNK signaling on lifespan is much less understood (Biteau et al., 2010). One of the most important contributions of this paper is the identification of an additive interaction between TOR and JNK pathways and demonstration that simultaneous inhibition of both produces longer rotifer lifespan and greater resistance to a variety of stressors than either alone. This could be an important finding since joint dosing of TOR and JNK inhibitors may allow them to be administered at lower doses, thereby avoiding some harmful side effects (Wilkinson et al., 2012).

A similar screening experiment for longevity effects was recently conducted on *Drosophila* males by Spindler et al. (2012) to test 80 small molecule inhibitors of mammalian receptor tyrosine kinases. These compounds were tested at 0.5 mM and 4.5 mM and 17 (21%) were observed to extend lifespan. Thirteen compounds targeted receptor tyrosine kinase signaling systems, including EGF, PDGF/VEGF, insulin/IGFI, JAK and GPC receptors and the downstream kinases p38, JNK and PKC. A subset of 11 compounds was tested on cultured *Drosophila* cells and 5 inhibited JNK, 3 activated JNK, and 3 caused no change in JNK expression. They also tested the same JNK inhibitor (SP600125) that we used in our experiments and found that 10 mM increased *Drosophila* male lifespan by 24%. In contrast, we exposed rotifer females to 1 µM SP600125 and observed 35% longer mean and median lifespan. These authors also exposed *Drosophila* males to everolimus, a rapamycin derivative, and found that 3 mM increased lifespan by 17%. In comparison, we exposed rotifers to 1 µM rapamycin and observed 35% longer mean lifespan, 29% longer median lifespan, and 53% longer maximum lifespan. Only two of the 12 inhibitors (16%) that we tested extended rotifer lifespan.

Also using a pharmacological approach, Bjedov et al. (2010) demonstrated that feeding rapamycin to adult *Drosophila melanogaster* mimics the life extension effect seen in some TOR mutants. Rapamycin-mediated life extension was not associated with alterations in either IIS or AMP-activated protein kinase (AMPK) activity, but was blocked by alterations to both autophagy and translation. Rapamycin exposure was associated with increased resistance to both starvation and

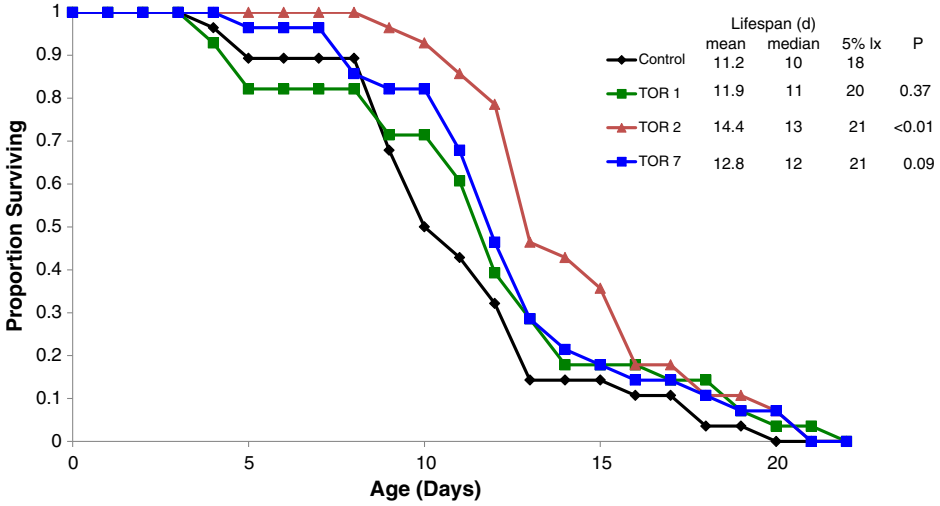

Fig. 10. Survival curves for *B. manjavacas* exposed to RNAi of the TOR gene. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. TOR 1, 2, and 7 are primers for different segments of the TOR gene sequence. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control.

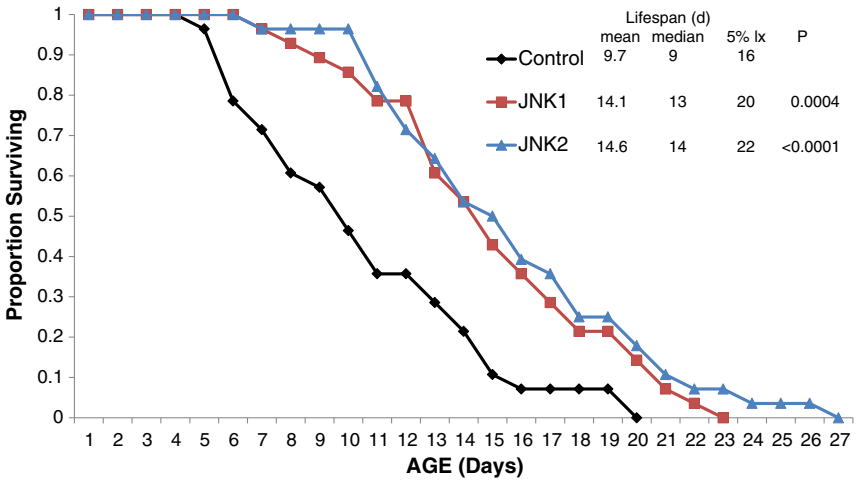

Fig. 11. Survival curves for *B. manjavacas* exposed to RNAi of the JNK gene. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. JNK 1 and 2 are primers for different segments of the JNK gene sequence. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control.

oxidative stressors. A 1 μM dose of rapamycin had no effect on the flies, but significant lifespan extension occurred at 50, 200, and 400 μM. Exposure to 200 μM rapamycin produced the largest increase in median lifespan, but female fecundity was severely reduced at this dose, which could account for some of the life extension effect. There was also a significant increase in the number of lysosomes after rapamycin treatment, suggesting an up-regulation of autophagy, potentially contributing to life extension. Dietary restriction did not further increase lifespan when TOR signaling was already reduced, suggesting that common mechanisms may mediate both interventions.

Our RNAi knockdown of TOR and JNK genes produced significant rotifer life extension similar to the chemical inhibition of these pathways. A major difference is that simultaneous RNAi knockdown of both TOR and JNK genes did not produce the additive effect observed by simultaneous exposure to rapamycin and JNK inhibitor. This may be explained if the inhibitors have a broader effect than just inhibition of the TOR and JNK genes. As can be seen in Table 1, rapamycin and JNK inhibitor affect associated proteins and downstream pathways in poorly understood ways. Perhaps these broader effects of rapamycin and JNK inhibitors contribute to bigger life extension effects than does the more narrowly targeted RNAi knockdown of single genes. Another possibility is that exposure to dsRNA of both TOR and JNK genes might reduce the efficiency of the RNAi knockdown process.

Treatments that produced rotifer life extension also conferred resistance to environmental stressors like starvation, UV, osmotic, oxidative, and high temperature stress. This linkage between aging and stress resistance has been described in a variety of contexts (e.g. Haigis and Yankner, 2010; Le Bourg, 2009; Miller, 2009; Rattan, 2008) and often has been linked to the general phenomenon of hormesis. Hormesis occurs when exposure to mild stress induces stress resistance pathways, conferring resistance to subsequent stressor exposures. We have observed hormesis in rotifers, but how this links to the slowing of aging is only just now being investigated. Moreover, the increased resistance to various stressors recorded in our study must have been induced by a different mechanism than hormesis. We did not pre-expose rotifers to mild stressors as in hormesis, we simply inhibited the TOR or JNK pathways and observed resistance to multiple stressors. Furthermore, often greater resistance was induced by the simultaneous inhibition of both TOR and JNK pathways, suggesting an interaction between them. The relationship between stress resistance by hormesis versus resistance by TOR and JNK pathway inhibition is not understood, but our observation suggests several new lines of investigation. These could include a survey of the pathways

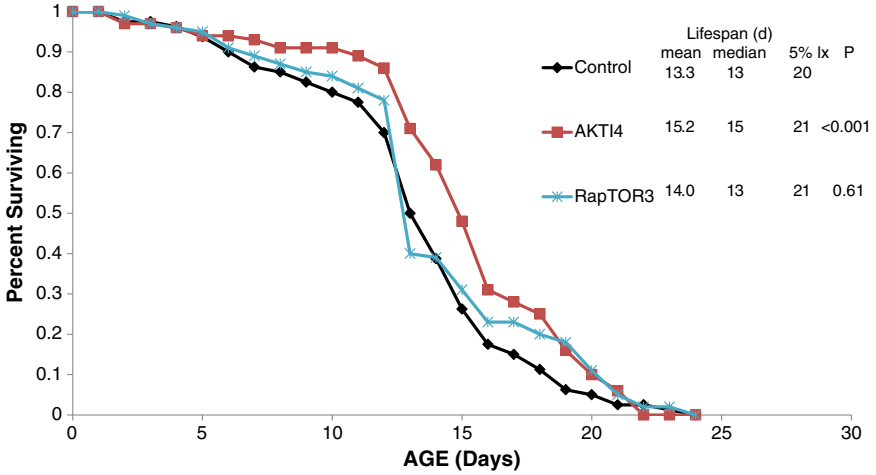

Fig. 12. Survival curves for *B. manjavacas* exposed to RNAi of the AKT and RapTOR genes. Proportion surviving represents the fraction of an initial cohort of 120 rotifers surviving to the indicated age. Mean, median and maximum lifespan (5% surviving) are reported in days. P refers to the Wilcoxon test probability that the treatment survival curves are similar to control.

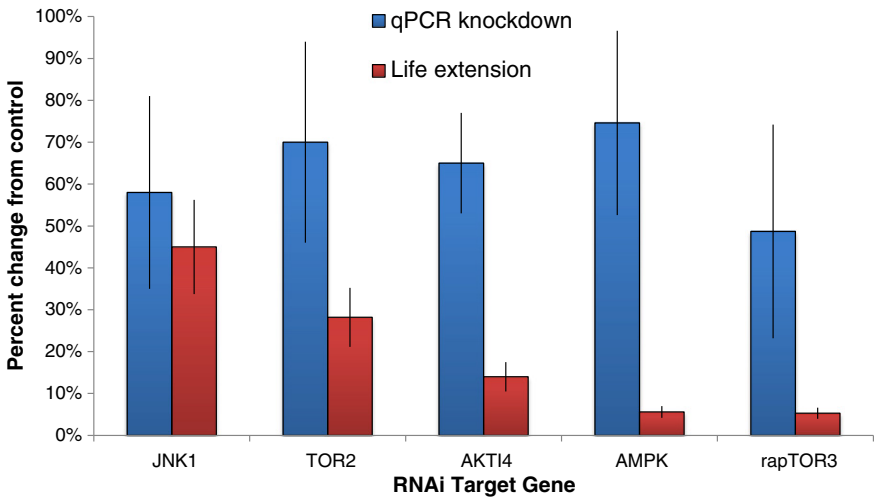

Fig. 13. Comparison of rotifer life extension by RNAi knockdown and reduction in gene expression estimated by qPCR. Rotifers were exposed to gene-specific dsRNA to induce RNAi and then survival curves characterized in a life table or gene expression estimated with qPCR. The Y-axis reports that the percent lifespan was extended following RNAi or the percentage of gene knockdown in qPCR relative to control. Asterisks indicate significant differences from the control. Vertical lines indicate standard error.

induced by hormesis, and whether the induced pathways include TOR and JNK or both, whether a schedule of continuous, weak induction of these pathways is more effective than intermittent, stronger induction, and what variability among these traits exists among rotifer populations and clades.

The differential effects of rapamycin and JNK inhibitor on the induction of stress resistance provide insight into the metabolic pathways involved. For example, exposure of rotifers to 1 μM rapamycin induced protection from starvation, but not UV, oxidative, osmotic or heat stress. This suggests altered expression of genes in nutrient sensing pathways (e.g. Hardie et al., 2012), but not UV stress (DNA repair pathways, Pines et al., 2013), oxidative stress (ROS scavengers like superoxide dismutase, Kowald, 2011), osmoregulatory stress (MAPK signaling cascades, Zhi et al., 2013), or the heat shock protein pathways (HSP90s, 70s, 60s, 40s and small HSPs, Richter et al., 2010). Similarly, exposure of rotifers to 1 μM JNK inhibitor conferred resistance only to starvation and osmotic stress, but exposure to both 0.5 μM rapamycin and JNK inhibitor expanded the protection to include starvation, osmotic and UV stressors. This additive effect emphasizes the cross-talk among pathways and illustrates how combined exposures can lower the therapeutic dose. Moreover, these observations permit hypotheses to be formulated about which pathways to focus upon in searching for molecular targets capable of life extension. Recent work on the transcriptional analysis of long-lived *Drosophila* has implicated stress resistance genes as one of the most important classes associated with extended longevity (Doroszuk et al., 2012).

Work on the rotifer *B. plicatilis* by Ozaki et al. (2010) demonstrated a link between caloric restriction (CR) and increased stress resistance. They showed that 3 day old rotifers starved on the second day, and 4 day old rotifers that starved on the second and fourth days were 26% and 52%, respectively, more resistant to hypoxia (<0.1% oxygen for 7.5 or 11 h) than ad libitum fed animals. They also showed using qPCR that the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and enolase were more highly expressed in the CR treatment. They concluded that CR enhances the glycolysis pathway in rotifers and this prepares the animals to more effectively resist the stress of hypoxia. This observation suggests an interesting hypothesis that treatments shifting metabolism from aerobic to anaerobic pathways could be as effective for life extension as CR. Extending this CR-stress resistance link to *Brachionus calyciflorus*, Yang et al. (2013) showed that CR increased expression of four heat shock genes (hsps 40, 60, 70, 90) and increased rotifer resistance to paraquat-induced oxidative stress.

The relationship between mitochondrial dysfunction and aging is poorly understood, although it is widely thought that the efficiency of oxidative phosphorylation diminishes with age, increasing electron leakage and reducing ATP generation (Green et al., 2011). Small molecules capable of conserving mitochondrial integrity would be of great interest for slowing aging. We found that exposure to 1 μM JNK inhibitor promoted about 2-fold greater mitochondrial activity in 4 day old rotifers than controls. Perhaps JNK inhibitor provides some protection from ROS damage, slowing oxidation of mitochondrial proteins and the destabilization of macromolecular complexes and mitochondrial membranes. However, we did not observe protection by JNK inhibitor of juglone-induced oxidative stress in our stress challenge experiments. Treatment with 1 μM rapamycin had no effect on rotifer mitochondrial activity.

Bjedov et al. (2010) demonstrated that feeding rapamycin to adult *D. melanogaster* significantly increased the number of lysosomes, suggesting an up-regulation of autophagy. Our observations in rotifers using the fluorescence intensity LysoTracker staining as a proxy for lysosome activity, found that lysosome activity increases to age 6 days, then declines in older age classes. This increase in lysosome activity corresponds to the ramping up to peak reproduction in *B. manjavacas*. Peak reproduction is likely associated with the highest metabolic rates and high levels of protein turnover (autophagy). In contrast to the findings in *Drosophila*, our observation was that rapamycin treatment diminished rotifer lysosome activity. Perhaps rapamycin treatment reduces the accumulation of protein damage in rotifers, thereby diminishing the level of autophagy.

In addition to mortality, rotifer swimming speed is another phenotype modified by aging. Swimming speed is an especially interesting variable because it is a good indicator of vitality in older age classes. Most aging researchers are not only interested in finding interventions that extend lifespan, but also in extending health span. *B. manjavacas* swim continuously after hatching, grazing on microalgae, such that their ingestion rate is proportional to their swimming speed. If they greatly slow their swimming speed, they sink in the water column out of the photic zone, which contains their food. Rotifer swimming is therefore an essential feature of their life history and a measure of their health and vitality. *B. manjavacas'* swimming speed peaks in middle age at about 2.5 mm/s and declines to 1 mm/s with senescence (Snell et al., 2012). Aged rotifers slow their swimming and eventually sink to the bottom of wells where they remain virtually immobile until death. Our observation that only the combined treatment of rapamycin and JNK inhibitor improved the swimming speed of 10 day old females, underscores the importance of the interaction between TOR and JNK pathways. Somehow simultaneous inhibition of both pathways enabled rotifers to resist the natural loss of swimming

speed with aging, whereas inhibition with single compounds could not. Elucidating the mechanism of this interaction seems like a promising line of future investigation.

Another promising area for investigation is a deeper analysis of how age-specific mortality rates change through the reproductive and post-reproductive phases in the various treatments. Our data allow us to determine whether the lifespan extension that we observed with rapamycin and JNK inhibitor treatments results from slower aging during reproduction or slower senescence rates during the post-reproductive phase. We plan to present this analysis in a follow-on paper.

Because of the multiplex nature of aging, perhaps the most effective strategy for extending healthy lifespan might be combinations of treatments capable of inhibiting multiple pathways simultaneously. Our results show that simultaneously inhibiting the TOR and JNK pathways produces additive effects. It therefore seems sensible to explore whether other treatments in combination with low doses of rapamycin and JNK inhibitor might also extend lifespan. These treatments may include supplements of certain amino acids, glycerol supplements, and periodic exposure to modestly lower temperatures (2/3 of ambient). We have observed that all of these treatments extend rotifer lifespan individually, but whether they have additive effects remains to be determined.

Conflict of interest

The authors have no conflicts of interests.

Acknowledgments

We acknowledge with gratitude the support of the National Institute of Aging, grant R01 AG037960-02 for this work. We also express our appreciation for the expert technical assistance provided by Joan Chung, Leilani Barry, and Corey Rennolds. David Mark Welch kindly provided the nucleotide sequences for the *B. manjavacas* TOR and JNK genes.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2014.01.022.

References

Apsel, B., Blair, J.A., Gonzalez, B., Nazif, T.M., Feldman, M.E., Aizenstein, B., Hoffman, R., Williams, R.L., Shokat, K.M., Knight, Z.A., 2008. Targeted polypharmacology: discovery of dual inhibitors of tyrosine and phosphoinositide kinases. Nat. Chem. Biol. 4 (11), 691–699. http://dx.doi.org/10.1038/nchembio.117.

Austad, S.N., 2009. Is there a role for new invertebrate models for aging research? J. Gerontol. A Biol. Sci. Med. Sci. 64A, 192–194.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. PNAS 98 (24), 13681–13686.

Biteau, B., Karpac, J., Hwangbo, D., Jasper, H., 2011. Regulation of *Drosophila* lifespan by JNK signalling. Exp. Gerontol. 46, 349–354.

Bjedov, I., Toivonen, J.M., Kerr, F., Slack, C., Jacobson, J., Foley, A., Partridge, L., 2010. Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. Cell Metab. 11, 35–46.

Dolez, M., Nicolas, J.-F., Hirsinger, E., 2011. Laminins, via heparan sulfate proteoglycans, participate in zebrafish myotome morphogenesis by modulating the pattern of Bmp responsiveness. Development 138, 97–106.

Doroszuk, A., Jonker, M.J., Pul, N., Breit, T.M., Zwaan, B.J., 2012. Transcriptome analysis of a long-lived natural *Drosophila* variant: a prominent role of stress- and reproduction-genes in lifespan extension. BMC Genomics 13, 167 (http://www.biomedcentral.com/1471-2164/13/167).

Dunn, C.W., Hejnol, A., Matus, D.Q., Pang, K., Browne, W.E., Smith, S.A., Seaver, E., Rouse, G.W., Obst, M., Edgecombe, G.D., Sorensen, M.V., Haddock, S.H.D., Rhaesa, A.S., Okusu, A., Kristensen, R.M., Wheeler, W.C., Martindale, M.Q., Giribet, G., 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. Nature 452, 745–749.

Edgecombe, G.D., Giribet, G., Dunn, C.W., Hejnol, A., Kristensen, R.M., Neves, R.C., Rouse, G.W., Worsaae, K., Sorensen, M.V., 2011. Higher level metazoan relationships: recent progress and remaining questions. Org. Divers. Evol. 11, 151–172.

Fontana, L., Partridge, L., Longo, V.D., 2010. Extending healthy lifespan—from yeast to humans. Science 328, 321–326.

Fontaneto, D., Giordani, I., Melone, G., Serra, M., 2007. Disentangling the morphological stasis in two rotifer species of the *Brachionus plicatilis* species complex. Hydrobiologia 583, 297–307.

García-Martínez, J.M., Moran, J., Clarke, R.G., Gray, A., Cosulich, S.C., Chresta, C.M., Alessi, D.R., 2009. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem. J. 421, 29–42. http://dx.doi.org/10.1042/BJ20090489.

Green, D.R., Galluzzi, L., Kroemer, G., 2011. Mitochondria and the autophagy–inflammation–cell death axis in organismal aging. Science 333, 1109–1112.

Gribble, K.E., Mark Welch, D.B., 2012. Lifespan extension by caloric restriction is determined by type and level of food reduction and by reproductive mode in *Brachionus manjavacas* (Rotifera). J. Gerontol. A Biol. Sci. Med. Sci. http://dx.doi.org/10.1093/gerona/gls170.

Guillard, R.R.L, 1983. Culture of phytoplankton for feeding marine invertebrates. In: Berg Jr., C.J. (Ed.), Culture of Marine Invertebrates. Hutchinson Ross, Stroudsburg, PA.

Haigis, M.C., Sinclair, D.A., 2010. Mammalian sirtuins: biological insights and disease relevance. Annu. Rev. Pathol. 5, 253–295.

Haigis, M.C., Yankner, B.A., 2010. The aging stress response. Mol. Cell 40, 333–344.

Hardie, D.G., Ross, F.A., Hawlet, S.A., 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. 13, 251–262. http://dx.doi.org/10.1038/nrm3311.

Hur, J.H., Cho, J., Walker, D.W., 2010. Aging: dial M for mitochondria. Aging 2, 69–73.

Jewell, J.L., Guan, K.L., 2013. Nutrient signalling to mTOR and cell growth. Trends Biochem. Sci. XX, 1–10.

Johnson, G.L., Nakamura, K., 2007. The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. Biochim. Biophys. Acta 1773, 1341–1348.

Kaeberlein, M., 2010. Lesson on longevity from budding yeasts. Nature 464, 513–519. http://dx.doi.org/10.1038/nature08981.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M.F., Fritz, L.C., Burrows, F.J., 2003. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425 (6956), 407–410.

Kapahi, P., Chen, D., Rogers, A.N., Katewa, S.D., Li, P.W., Thomas, E.L., Kockel, L., 2010. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab. 11, 453–465.

Karpac, J., Jasper, H., 2009. Insulin and JNK: optimizing metabolic homeostasis and lifespan. Trends Endocrinol. Metab. 20, 100–106.

Karpac, J., Hull-Thompson, J., Falleur, M., Jasper, H., 2009. JNK signaling in insulin-producing cells is required for adaptive responses to stress in *Drosophila*. Aging Cell 8, 288–295.

Katewa, S.D., Kapahi, P., 2011. Role of TOR signaling in aging and related biological processes in *Drosophila melanogaster*. Exp. Gerontol. 46, 382–390.

Kim, J.I., Lee, H.R., Sim, S.E., Baek, J., Yu, N.K., Choi, J.H., Ko, H.G., Lee, Y.S., Park, S.W., KwakC, AhnSJ, Choi, S.Y., Kim, H., Kim, K.H., Backx, P.H., Bradley, C.A., Kim, E., Jang, D.J., Lee, K., Kim, S.J., Zhuo, M., Collingridge, G.L., Kaang, B.K., 2011. PI3K [gamma] is required for NMDA receptor-dependent long-term depression and behavioral flexibility. Nat. Neurosci. 14 (11), 1447–1454.

Kim, R.O., Rhee, J.S., Won, E.J., Lee, K.W., Kang, C.M., Lee, Y.M., Lee, J.S., 2011. Ultraviolet B retards growth, induces oxidative stress, and modulates DNA repair-related gene and heat shock protein gene expression in the monogonont rotifer, *Brachionus* sp. Aquat. Toxicol. 101, 529–539.

Kowald, A., 2011. The glyoxalase system as an example of a cellular maintenance pathway with relevance to aging. Aging 3, 17–18.

Le Bourg, E., 2009. Hormesis, aging and longevity. Biochim. Biophys. Acta 1790, 1030–1039.

Lee, K.S., Iijima-Ando, K., Iijima, K., Lee, W.J., Lee, J.H., Yu, K., Lee, D.S., 2009. JNK/FOXOmediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends life span. J. Biol. Chem. 284, 29454–29461.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2-\Delta\Delta Ct$ method. Methods 25, 402–408.

Lopez-Otın, C., Blasco, M.A., Partridge, L., Serrano, M., Kroemer, G., 2013. The hallmarks of aging. Cell 153, 1194–1217.

Magalhaes, J.P., Wuttke, D., Wood, S.H., Plank, M., Vora, C., 2012. Genome–environment interactions that modulate aging: powerful targets for drug discovery. Pharmacol. Rev. 64, 88–101.

McCormick, M.A., Tsai, S.Y., Kennedy, B.K., 2011. TOR and aging: a complex pathway for a complex process. Phil. Trans. R. Soc. B 366, 17–27.

Miller, R.A., 2009. Cell stress and aging: new emphasis on multiplex resistance mechanisms. J. Gerontol. A Biol. Sci. Med. Sci. 64A, 179–182.

Ozaki, Y., Kaneko, G., Yanagawa, Y., Watabe, S., 2010. Calorie restriction in the rotifer *Brachionus plicatilis* enhances hypoxia tolerance in association with the increased mRNA levels of glycolytic enzymes. Hydrobiologia 649, 267–277.

Pines, A., Mullenders, L.H., van Attikum, H., Luijsterburg, M.S., 2013. Touching base with PARPs: moonlighting in the repair of UV lesions and double-strand breaks. Trends Biochem. Sci. 38, 321–330.

Preston, B.L., Kneisel, R.A., Snell, T.W., 1999. Effects of UV–B irradiation on the toxicant sensitivity of the rotifer *Brachionus calyciflorus*. Environ. Pollut. 106, 23–31.

Raible, F., Arendt, D., 2004. Metazoan evolution: some animals are more equal than others. Curr. Biol. 14, 106–108.

Rattan, S.I.S., 2008. Hormesis in aging. Aging Res. Rev. 7, 63–78.

Rhee, J.S., Kim, R.O., Kim, B.M., Dahms, H.U., Lee, J.S., 2012. Genomic organization of selected genes in the small monogonont rotifer, *Brachionus koreanus*. Gene 505, 108–113.

Richter, K., Haslbeck, M., Buchner, J., 2010. The heat shock response: life on the verge of death. Mol. Cell 40, 253–266.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., Sabatini, D.M., 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell 22, 159–168.

Sengupta, S., Peterson, T.R., Sabatini, D.M., 2010. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol. Cell 40, 310–322.

Sharp, Z.D., 2011. Aging and TOR: interwoven in the fabric of life. Cell. Mol. Life Sci. 68, 587–597.

Shore, D.E., Ruvkun, G., 2013. A cytoprotective perspective on longevity regulation. Trends Cell Biol. XX, 1–12. http://dx.doi.org/10.1016/j.tcb.2013.04.007.

Skold, H.K., Obst, M., 2011. Potential for clonal animals in longevity and aging studies. Biogerontology 12, 387–396.

Snell, T.W., 2014. Rotifers as models for the biology of aging. Int. Rev. Hydrobiol. (in press).

Snell, T.W., Shearer, T.L., Smith, H.A., 2011. Exposure to dsRNA elicits RNA interference in *Brachionus manjavacas* (Rotifera). Mar. Biotechnol. 13, 264–274.

Snell, T.W., Fields, A.M., Johnston, R.K., 2012. Antioxidants can extend lifespan of *Brachionus manjavacas* (Rotifera), but only in a few combinations. Biogerontology 7. http://dx.doi.org/10.1007/s10522-012-9371-x.

Spindler, S.R., Li, R., Dhahbi, J.M., Yamakawa, A., Sauer, F., 2012. Novel protein kinase signaling systems regulating lifespan identified by small molecule library screening using *Drosophila*. PLoS One 7 (2), e29782. http://dx.doi.org/10.1371/journal.pone.0029782.

Stanfel, M.N., Shamieh, L.S., Kaeberlein, M., Kennedy, B.K., 2009. The TOR pathway comes of age. Biochim. Biophys. Acta 1790 (2009), 1067–1074.

Takeuchi, H., Kondo, Y., Fujiwara, K., Kanzawa, T., Aoki, H., Mills, G.B., Kondo, S., 2005. Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. Cancer Res. 65 (8), 3336–3346.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., Gray, N.S., 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J. Biol. Chem. 284, 8023–8032.

Twumasi-Boateng, K., Wang, T.W., Tsai, L., Lee, K.-H., Salehpour, A., Bhat, S., Tan, M.-W., Shapira, M., 2012. An age-dependent reversal in the protective capacities of JNK signalling shortens *Caenorhabditis elegans* lifespan. Aging Cell 11, 659–667.

Ungvari, Z., Philipp, E.E.R., 2011. Comparative gerontology—from mussels to man. J. Gerontol. A Biol. Sci. Med. Sci. 66A (3), 295–297.

Wang, M.C., Bohmann, D., Jasper, H., 2005. JNK extends lifespan and inhibits growth by antagonizing cellular and organism-wide responses to insulin signalling. Cell 121, 115–125.

Weston, C.R., Davis, R.J., 2007. The JNK signal transduction pathway. Curr. Opin. Cell Biol. 19, 142–149.

Wilkinson, J.E., Burmeister, L., Brooks, S.V., Chan, C.C., Friedline, S., Harrison, D.E., Hejtmancik, J.F., Nadon, N., Strong, R., Wood, L.K., et al., 2012. Rapamycin slows aging in mice. Aging Cell 11, 675–682.

Yang, J., Mu, Y., Dong, S., Jiang, Q., Yang, J., 2013. Changes in the expression of four heat shock proteins during the aging process in *Brachionus calyciflorus* (Rotifera). Cell Stress Chaperones. http://dx.doi.org/10.1007/s12192-013-0432-0.

Yu, K., Shi, C., Toral-Barza, L., Lucas, J., Shor, B., Kim, J.E., Zhang, W.G., Mahoney, R., Gaydos, C., Tardio, L., Kim, S.K., Conant, R., Curran, K., Kaplan, J., Verheijen, J., Ayral-Kaloustian, S., Mansour, T.S., Abraham, R.T., Zask, A., Gibbons, J.J., 2010. Beyond rapalog therapy: preclinical pharmacology and antitumor activity of WYE-125132, an ATP-competitive and specific inhibitor of mTORC1 and mTORC2. Cancer Res. 70 (2), 621–631. http://dx.doi.org/10.1158/0008-5472.CAN-09-2340.

Yuan, J.S., Reed, A., Chen, F., Stewart Jr., C.N., 2006. Statistical analysis of real-time PCR data. BMC Bioinforma. 7, 85.

Yuan, H.X., Xiong, Y., Guan, K.L., 2013. Nutrient sensing, metabolism, and cell growth control. Mol. Cell 49, 379–387.

Zhi, H., Tang, L., Xia, Y., Zhang, J., 2013. Ssk1p-Independent activation of Ssk2p plays an important role in the osmotic stress response in *Saccharomyces cerevisiae*: alternative activation of Ssk2p in osmotic stress. PLoS One 8 (2), e54867. http://dx.doi.org/10.1371/journal.pone.0054867.